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Rapid and Simple Approach for Identification of *Mycobacterium tuberculosis* Complex Isolates by PCR-Based Genomic Deletion Analysis

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Although the virulences and host ranges differ among members of the *Mycobacterium tuberculosis* complex (TBC; *M. tuberculosis*, *M. africanum*, *M. canettii*, *M. microti*, *M. bovis*, and *M. bovis* BCG), commercially available molecular assays cannot differentiate these organisms because of the genetic identities of their 16S rRNA gene sequences. Comparative genomic analyses with the complete DNA sequence of *M. tuberculosis* H37Rv has provided information on regions of difference (RD 1 to RD 16) deleted in members of the TBC other than *M. tuberculosis*. To determine whether deletion analysis could accurately differentiate members of TBC, we used PCR to assess the presence or absence of specific regions of the genome in 88 well-characterized isolates of *M. tuberculosis*, *M. africanum*, *M. microti*, *M. bovis*, and *M. bovis* BCG. The identifications obtained by use of the specific deletion profiles correlated 100% with the original identifications for all TBC members except *M. africanum*, but further characterization resulted in profiles specific for all members. Although six RD regions were used in the analyses with the original 88 isolates, it was found that the use of RD 1, RD 9, and RD 10 was sufficient for initial screenings, followed by the use of RD 3, RD 5, and RD 11 if the results for any of the first three regions were negative. When 605 sequential clinical isolates were screened, 578 (96%) were identified as *M. tuberculosis*, 6 (1%) were identified as *M. africanum*, 8 (1%) were identified as *M. bovis*, and 13 (2%) were identified as *M. bovis* BCG. Since PCR-based assays can be implemented in most clinical mycobacteriology laboratories, this approach provides a rapid and simple means for the differentiation of members of TBC, especially *M. bovis* and *M. tuberculosis*, when it is important to distinguish between zoonotic sources (i.e., cattle and unpasteurized dairy products) and human sources of tuberculosis disease.

The *Mycobacterium tuberculosis* complex (TBC) (4, 34) comprises the closely related organisms *M. tuberculosis*, *M. africanum*, *M. bovis*, the *M. bovis* BCG vaccine strain, and two rarely seen members, *M. microti* and *M. canettii* (35). Differentiation of the members of the TBC is necessary for the treatment of individual patients and for epidemiological purposes, especially in areas of the world where tuberculosis has reached epidemic proportions or wherever the transmission of *M. bovis* between animals or animal products and humans is a problem. In addition, it can be important to rapidly identify isolates of *M. bovis* BCG recovered from immunocompromised patients.

Although no clear-cut means of differentiation of the members of the TBC was found in the past by using numerical classification (34), a few conventional methods have been useful. Those methods include assays for the ability to metabolize glycerol or pyruvate in Loewenstein-Jensen medium, oxygen preference (aerophilic versus microaerophilic), niacin accumulation, nitrate reductase activity, colony morphology, and resistance to two compounds, thiophen-2-carboxylic acid hydrazide (TCH) and pyrazinamide (PZA) (12, 19, 38). Partially due to the slow growth of the TBC, interpretation of the results of these assays can be highly subjective, especially interpretation of differences in colony morphology (19), which can be due to the loss of virulence or to mutations associated with drug resistance. An alternative approach is the use of high-performance liquid chromatography; however, only the profile for *M. bovis* BCG differs from those for the other members of the complex (10).

Testing for resistance to TCH has been reported to be the only single test that assigned isolates to any specific member of the TBC; classical *M. tuberculosis* isolates are resistant to TCH, irrespective of their resistance to isoniazid (8). Alternatively, the Asian strain of *M. tuberculosis* and all other members of the TBC are TCH susceptible (39, 40). However, cross-resistance to TCH has been seen in isolates of *M. bovis* expressing resistance to high levels of isoniazid (0.4 μg/ml) (L. M. Parsons, unpublished observations, 2001). Multidrug-resistant TBC isolates can be resistant to PZA, but resistance only to PZA (monodrug resistance) is found almost exclusively in *M. bovis* and *M. bovis* BCG (12, 38; M. Salfinger, L. B. Reller, and F. M. Kafader, Abstr. 90th Annu. Meet. Am. Soc. Microbiol. 1990, abstr. U-55, p. 150, 1990). Because *M. africanum* has a phenotype intermediate between those of *M. tuberculosis* and *M. bovis* and is susceptible to PZA, this member of the TBC was

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TABLE 1. Genetic differences among members of the TBC

Component evaluated	Difference	Reference(s)
Variable alleles		
oxyR nucleotide 285	A in *M. bovis*, G in other members of the TBC	30
pncA nucleotide 169	G in *M. bovis* and *M. bovis* BCG, C in other members of the TBC	28
katG codon 463	CTG (Leu) in group 1, CGG (Arg) in group 2, CGG (Arg) in group 3[a]	31
gyrA codon 95	ACC (Thr) in group 1, ACC (Thr) in group 2, AGC (Ser) in group 3[a]	
gyrB	Sequence differences among members of the TBC	17, 25
Variable sequences for spacers between direct repeats[b]		
Spacers 33 to 36 (derived from BCG)	*M. tuberculosis* does not hybridize to the spacers	16
Spacers 39 to 43 (derived from *M. tuberculosis*)	*M. bovis* and BCG do not hybridize to the spacers	16
Spacers 37 and 38	*M. microti* has a very short direct repeat region; many strains only hybridize to spacers 37 and 38	36
Spacers 8, 9 and 39	*M. africanum* does not hybridize to the spacers	37

[a] Group 1 contains *M. tuberculosis, M. africanum, M. microti*, and *M. bovis*; groups 2 and 3 contain only *M. tuberculosis*.
[b] As determined by spoligotyping.

originally called *M. bovis*, Afro-Asian variety (23). However, difficulties in the precise definition of *M. africanum* were further complicated when variants associated with different geographic regions were described (variant I was an *M. bovis*-like organism that was nitrate negative and that was from West Africa; variant II was an *M. tuberculosis*-like organism that was nitrate positive and that was from East Africa) (7). A more recent study from West Africa suggested that similar numbers of the two variants are present in the population in Guinea-Bissau (14).

In DNA-DNA hybridization assays, the four members of the TBC tested were found to share 85 to 100% DNA-DNA relatedness (15). Subsequently, completely conserved DNA sequences were reported for the 16S rRNA gene (rDNA) and 16S-23S rDNA spacers (20). Furthermore, no significant nucleotide sequence variations either in 26 structural genes or in 24 genes coding for proteins that are targets of the host immune system were found among a diverse group of isolates of *M. tuberculosis* (24, 31).

Unfortunately, the high degree of sequence conservation among the members of the TBC has resulted in difficulties for the clinical mycobacteriology laboratory since commercial DNA probe and amplification assays based on 16S rDNA sequences, identical for all TBC members, cannot be used to differentiate members of the complex. Further complications have arisen following the addition to the complex of three recently described members that are positive by commercial hybridization assays: *M. canettii* (35), *M. tuberculosis* subsp. *caprae* (1), and the unnamed seal bacillus (41).

Despite the difficulties described above, advances in molecular methods and the accumulating knowledge of the *M. tuberculosis* genome have resulted in methods designed to rapidly identify the members of the TBC. These methods are based on differences in various alleles and repetitive regions, mutations associated with drug resistance, and transposition of mobile elements (Table 1). While these variations in molecular characteristics have enabled scientific distinctions to be made between the different members of the complex, the complexities of the methods have hindered development of a single, direct assay for rapid identification.

Recently, comparative genomics with the complete DNA sequence of *M. tuberculosis* H37Rv has resulted in the demonstration of 16 regions of the genome (regions of difference [RD]) deleted in *M. bovis* and *M. bovis* BCG; subsequent studies found that some of these regions are also deleted in other members of the TBC (2, 3, 4, 11, 22). On the basis of these data, the aim of the present study was to investigate whether molecular amplification methods for determination of the presence or absence of specific regions of the genome could be used by clinical laboratories as rapid and simple assays for the precise identification of individual members of the TBC. This approach was validated with 88 well-characterized TBC isolates and was then used to identify 605 members of the TBC recovered from clinical specimens from March 2000 through June 2001.

MATERIALS AND METHODS

Bacterial isolates. In the first phase of the study, 88 members of the TBC were obtained from specimens submitted to the following laboratories: the Clinical Mycobacteriology Laboratory, Wadsworth Center, Albany, N.Y.; the Armauer Hansen Institute, German Leprosy Relief Association, Würzburg, Germany; the National Institute of Public Health and the Environment, Bilthoven, The Netherlands; and the Florida Department of Health, Jacksonville. In addition, strains of *M. africanum* and *M. microti* were purchased from the American Type Culture Collection. Each laboratory that submitted isolates confirmed that the isolates belonged to the TBC by commercial nucleic acid amplification or hybridization methods, with the final identification based on conventional phenotypic methods (12, 19, 38). Once the study protocol was established with the 88 previously identified isolates, the additional 605 isolates belonging to the TBC were obtained from specimens submitted to the Wadsworth Center from March 2000 through June 2001.

PCR deletion analyses. The genomes of the isolates were analyzed by PCR for the presence or the absence of six regions (RD 1, RD 3, RD 5, RD 9, RD 10, and RD 11) originally described as being deleted in the genomes of BCG isolates relative to the sequence of *M. tuberculosis* H37Rv (2, 3, 11, 22). A multiprimer PCR assay with three primers was used to detect RD 1, RD 9, and RD 10. In these assays, two primers complementary to sequences flanking the deleted region amplified a small product from strains from which the region was deleted. A third primer complementary to internal sequences and one of the flanking primers amplified a product of a different size when the region was present since the two flanking primers are too far apart to efficiently amplify the entire region for those strains. The primer sequences and PCR product sizes are listed in Table 2, with the sequence of the primer for RD 1 (9,455 bp) taken from that used in the previously published RD 1 multiprimer assay (32). The sequences for the primers for RD 9 (2,030 bp) and RD 10 (1,903 bp) were determined for this study by retrieving the sequences of the deleted regions (Sequence Retrieval Service, Institut Pasteur [www.srs.pasteur.fr]; accession numbers, Y181604 and AJ131209 for RD 9 and RD 10, respectively) and the flanking sequences for these two regions published by Gordon and coworkers (11). Primers were selected by importing the sequences into the Primer 3 program at www.genome.wi.mit.edu. For these three assays, the 50-μl reaction mixture contained each of the two flanking primers at a concentration of 10 mM, 50 mM internal primer, 50 mM

TABLE 2. Primer sequences for deletion analyses

Region and primer	Sequence
RD 1 (region present, 150 bp; region absent, 200 bp)	
ET1	5′-AAG-CGG-TTG-CCG-CCG-ACC-GAC-C-3′
ET2	5′-CTG-GCT-ATA-TTC-CTG-GGC-CCG-G-3′
ET3	5′-GAG-GCG-ATC-TGG-CGG-TTT-GGG-G-3′
RD 3 (region present, 500 bp; region absent, no product)	
RD3intF	5′-TTA-TCT-TGG-CGT-TGA-CGA-TG-3′
RD3intR	5′-CAT-ATA-AGG-GTG-CCC-GCT-AC-3′
RD 5 (region present, 152 bp; region absent, no product)	
mtp40F	5′-CTG-GTC-GAA-TTC-GGT-GGA-GT-3′
mtp40R	5′-ATG-GTC-TCC-GAC-ACG-TTC-GAC-3′
RD 9 (region present, 306 bp; region absent, 206 bp)	
RD9 FF	5′-GTG-TAG-GTC-AGC-CCC-ATC-C-3′
RD9 Int	5′-CAA-TGT-TTG-TTG-CGC-TGC-3′
RD9 FR	5′-GCT-ACC-CTC-GAC-CAA-GTG-TT-3′
RD 10 (region present, 308 bp; region absent, 202 bp)	
RD10 FF	5′-CTG-CAA-CCA-TCC-GGT-ACA-C-3′
RD10 Int	5′-GAA-GTC-GTA-ACT-CAC-CGG-GA-5′
RD10 FR	5′-AAG-CGC-TAC-ATC-GCC-AAG-3′
RD 11 (region present, 454 bp; region absent, no product)	
RD11intF	5′-CGG-CAG-CTA-GAC-GAC-CTC-3′
RD11intR	5′-AAC-GTG-CTG-CGA-TAG-GTT-TT-3′

KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.01% gelatin, each deoxynucleoside triphosphate at a concentration of 200 μM, 1.25 U of *Taq* DNA polymerase, and 10 μl of heat-killed (1 h at 80°C) bacterial cells. After denaturation at 95°C for 5 min, the reaction mixtures were incubated for 40 cycles at 94°C for 30 s and 1 min at 65°C, followed by 10 min at 72°C, in a GeneAmp 9700 (Perkin-Elmer Biosystems, Foster City, Calif.). Fifteen-microliter samples were run at 125 V for 1 h on a 2% agarose gel, and the band size was estimated by comparison to a 1-kb-plus DNA ladder (Gibco BRL, Life Technologies, Gaithersburg, Md.).

Because RD 5 is flanked by a repetitive region and RD 3 and RD 11 contain potentially mobile bacteriophages, only internal primers were used for the detection of these regions. The assay for RD 5 amplified a 152-bp product from *mtp40* (*plcA*), as described previously (33). The primers for RD 3 and RD 11 were obtained for this study by using the Primer 3 program from the integrase genes from lysogenic bacteriophages ϕRv1 and ϕRv2, respectively. The primers, listed in Table 2, were used to amplify 500- and 454-bp products when RD 3 (ϕRv1) and RD 11 (ϕRv2) were present. For these three assays, the same reaction components described above were used, but with different cycling temperatures and times. For RD 5, the samples were denatured at 95°C for 5 min and were then incubated at 94°C for 30 s, 64°C for 30 s, and 30 s at 72°C for 40 cycles, followed by 10 min at 72°C. For RD 3 and RD 11, following denaturation at 95°C for 5 min, the samples were incubated at 95°C for 1 min, 55°C for 1 min, and 1 min at 72°C for 40 cycles, followed by 10 min at 72°C. The products were analyzed as described above.

Phenotypic characterization. Drug resistance assays were performed by standard radiometric procedures with the BACTEC instrument and included assays for resistance to 100 μg of PZA per ml and 1 μg of TCH per ml (13, 27, 29). The PZA assay was completed at the time when the growth index (GI) for the control vial was equal to or greater than 200. If the GI for the PZA-containing vial was greater than 10% of that for the control vial at that time, the isolate was considered resistant to PZA. Resistance to TCH was noted when a steady increase in the GI for the TCH-containing vial was greater than the increase seen for a control vial with no drug. In previous studies, 5 μg of TCH per ml was used in assays with solid medium, whereas 1 to 2 μg of TCH per ml was used in the broth-based radiometric test (8, 29).

An oxygen preference assay (12, 21) was performed by inoculating 0.2 ml of an actively growing bacterial suspension into 10 ml of Middlebrook 7H9 broth containing 0.2% agar (in a screw-cap tube). The location of the growth in this semisolid medium was evaluated after 3 weeks of incubation at 37°C. A preference for aerophilic conditions was suggested by growth on the surface or no more than 5 mm below the surface of the medium. A preference for microaerophilic conditions was suggested by a band of growth approximately 10 to 15 mm below the surface. The percentage of agar used in the oxygen preference assay was increased to 0.2% from the 0.1% described previously (12) because the results could not be clearly visualized when the lower percentage was used. The use of 0.2% agar resulted in a clear distinction between aerophilic and microaerophilic growth. Additional assays included tests for niacin accumulation and nitrate reductase, performed by standard methods with organisms from a 2- to 3-week-old growth on Loewenstein-Jensen medium (19).

RESULTS

Results of deletion analyses with 88 previously characterized isolates. At the time when this study began, 16 regions had been found to be deleted in *M. bovis* BCG relative to the sequence of *M. tuberculosis* H37Rv (2, 3, 11, 22). It was shown that some of these regions were also absent from other members of the TBC, thus suggesting that the presence or absence of these regions could be useful in differentiation of members of the complex. For this study, six regions from various locations in the chromosome were selected for use as possible identification tools. The regions were RD 1, absent only in *M. bovis* BCG; RD 5, present in most strains of *M. tuberculosis*, *M. africanum*, and *M. microti* but absent in *M. bovis* and *M. bovis* BCG; RD 3 and RD 11, lysogenic bacteriophages found to be useful in differentiating *M. bovis* and *M. microti*; and RD 9 and RD 10, absent in isolates of *M. africanum* (2, 3, 4, 11, 22).

Typical reactions demonstrating the presence or absence of the six RD regions are shown in Fig. 1, with the results for the 88 previously characterized strains listed in Table 3. The 27 strains of *M. tuberculosis* were all positive (100%) for RD 1, RD 9, RD 10, and RD 11; most (96%) were positive for RD 5; and there were variable results for RD 3 (26%). The 25 strains previously identified as *M. africanum* were more genetically diverse, with 100% positivity seen only for RD 1. However, 6 of the 25 strains were positive for all six regions, and further analysis of representative strains (see below) resulted in a phenotype consistent with that of *M. tuberculosis*. When the results for the 19 other *M. africanum* strains were evaluated alone, a distinct profile was seen (Table 3): RD 1 was always present and RD 9 was always absent, with variable results obtained for the other regions. Although only five strains of *M. microti* were analyzed, a distinct profile was seen for these strains: all were positive for RD 1 and RD 11, variable for RD 5 (60%), and negative for RD 3, RD 9, and RD 10. The 14 *M. bovis* strains also contained RD 1, were often positive for RD 3 (71%), and were negative for RD 5, RD 9, RD 10, and RD 11. Finally, all six regions were absent in the 17 BCG strains tested.

Selected phenotypic assays. Because there was some overlap in the deletion profiles, assays for the detection of resistance to TCH and PZA and oxygen preference were added to confirm the identifications for the isolates. Although the Asian strain of

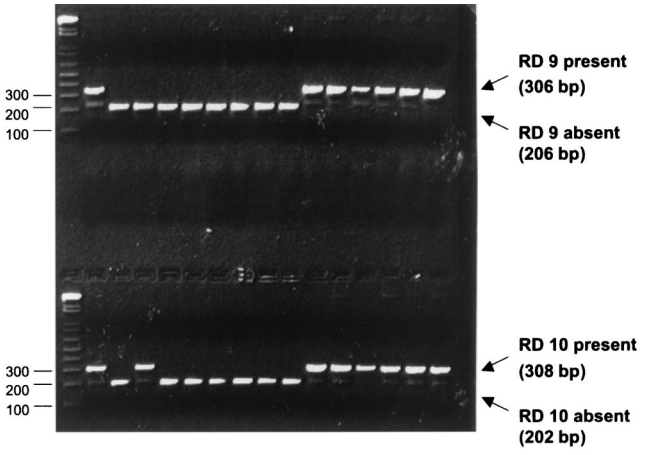

FIG. 1. Results of PCR for presence of RD 9 and RD 10. The larger PCR products indicate the presence of RD 9 (upper lanes) or RD 10 (lower lanes), while the smaller products indicate that the region has been deleted. The size standards (in base pairs) are labeled on the left side of the same 2% agarose gel prepared with a double comb.

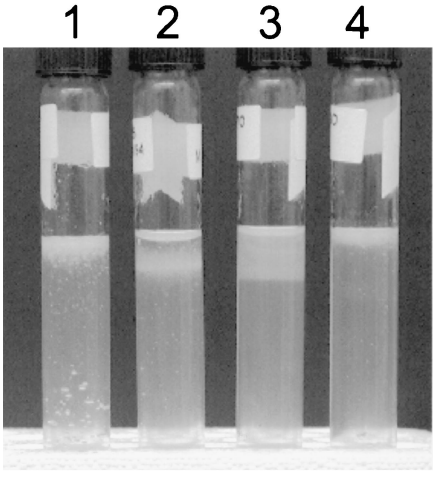

FIG. 2. Oxygen preference test (Middlebrook 7H9 broth with 0.2% agar). Four different members of the *M. tuberculosis* complex are shown, with the oxygen preference typical for each member indicated. Tube 1, *M. tuberculosis*; tube 2, *M. africanum*; tube 3, *M. bovis*; tube 4, *M. bovis* BCG.

M. tuberculosis can be TCH susceptible, previous classification schemes have listed only classical *M. tuberculosis* isolates as TCH resistant; likewise, monodrug resistance to PZA has been listed only for *M. bovis* and *M. bovis* BCG, and a preference for microaerophilic conditions differentiates *M. africanum* and *M. bovis* from the other members of the complex (Fig. 2) (12, 19, 38).

These three assays were used to test viable representatives of the original 88 isolates. The following results were consistent for each member of the TBC, as shown in Table 3: *M. tuberculosis*, TCH resistant, no monodrug resistance to PZA, preference for aerophilic conditions; *M. microti*, TCH susceptible, no monodrug resistance to PZA, preference for aerophilic conditions; *M. bovis*, TCH susceptible, monodrug resistance to PZA, preference for microaerophilic conditions; and *M. bovis* BCG, TCH susceptible, monodrug resistance to PZA, preference for aerophilic conditions.

For the group of isolates that were previously identified as *M. africanum*, monodrug resistance to PZA was not seen; however, variable results were obtained in the assays for resistance to TCH and oxygen preference. The 19 isolates that were negative for RD 9 were consistently susceptible to TCH and preferred microaerophilic conditions. However, two viable representatives of the six isolates that were positive for all RD regions were aerophilic, with one isolate being resistant to TCH and the other being susceptible. These isolates were found to be positive for niacin and negative for nitrate. The original identification was based solely on the phenotype; however, some phenotypic assays are variable for both *M. tuberculosis* and *M. africanum*. In some instances, strains of *M. tuberculosis* can be susceptible to TCH or have a negative reaction for nitrate reductase, phenotypes that are usually listed for *M. africanum*. Thus, we propose that a more precise differentia-

TABLE 3. Percentages of the 88 members of TBC that contain the six RD regions and selected phenotypes of the strains

RD region or phenotype	% of isolates					
	M. tuberculosis (27)[a]	*M. africanum*[b] (25)	*M. africanum*[c] (19)	*M. microti* (5)	*M. bovis* (14)	BCG (17)
RD 1[d]	**100**	100	**100**	**100**	**100**	0
RD 3	26	32	11	0	71	0
RD 5	96	96	95	60	0	0
RD 9	**100**	24	**0**	**0**	**0**	**0**
RD 10	**100**	56	**42**	**0**	**0**	**0**
RD 11	100	56	42	100	0	0
Monodrug resistance to PZA	0	0	0	0	100	100
Resistance to TCH	100	4	0	0	0	0
Preference for O_2[e]	A	A or M	M	A	M	A
Niacin positive	93	90	88	ND[f]	0	0
Nitrate positive	86	0	0	ND	0	25

[a] The values in parentheses are the numbers of isolates.
[b] Six of the 25 isolates originally identified as *M. africanum* were positive for all six regions; however, two of the six were found to phenotypically resemble *M. tuberculosis* upon retesting.
[c] The six isolates resembling *M. tuberculosis* phenotypically were omitted from this group.
[d] The boldface indicates the three regions selected for initial screening.
[e] A, aerophilic growth preferred; M, microaerophilic growth preferred.
[f] ND, not done.

TABLE 4. Results from screening of 605 clinical isolates belonging to TBC

Profile[a]	Identification	No. (%) of isolates			Oxygen preference[b]
		Total	TCH resistant	Resistant only to PZA	
A	*M. tuberculosis*	578	517 (89)	0	Aerophilic (87/90 [97])
B	*M. africanum*	6	0	0	Microaerophilic (6/6 [100])
C	*M. bovis*	8	0	8 (100)	Microaerophilic (8/8 [100])
D	*M. bovis* BCG	13	0	13 (100)	Aerophilic (13/13 [100])

[a] A, positive for RD 1, RD 9, RD 10 (tests for RD 3, RD 5, and RD 11 were not done); B, positive for RD 1, negative for RD 9, and variable for RD 3, RD 5, RD 10, and RD 11; C, positive for RD 1, variable for RD 3, and negative for RD 5, RD 9, RD 10, and RD 11; D, negative for all six regions.
[b] The values in parentheses indicate the number of isolates with the indicated oxygen reference/total number of isolates tested (percent).

tion of the members of the TBC should include an analysis for the presence or absence of these RD regions, and for an isolate to be identified as *M. africanum*, RD 9 should be absent.

Use of deletion analyses for screening of 605 clinical isolates. For the screening of the 605 clinical isolates obtained from a diverse patient population in New York State including New York City, the following strategy was used. After detection of growth of a pure culture of TBC in a BACTEC 12B vial, PZA and TCH were inoculated as part of the routine susceptibility testing. In addition, a portion of the bacterial suspension was heat killed and PCR assays for RD 1, RD 9, and RD 10 were performed. If any of the regions were absent, PCR assays for RD 3, RD 5, and RD 11 were performed. If the organism was susceptible to TCH, an oxygen preference assay was inoculated. Some TCH-resistant isolates were also tested for oxygen preference.

Table 4 summarizes the results of these assays. Of the 605 isolates, 578 (96%) were identified as *M. tuberculosis*, 6 (1%) were identified as *M. africanum*, 8 (1%) were identified as *M. bovis*, and 13 (2%) were identified as *M. bovis* BCG.

Of the 578 isolates identified as *M. tuberculosis* on the basis of the presence of RD 1, RD 9, and RD 10, 89% were the "classical" human strain resistant to TCH, 11% were the TCH-susceptible Asian strain, and none were drug resistant only to PZA. There were slight differences in the percentages of niacin- and nitrate-positive isolates in the two groups. Among the isolates of the classical strain tested, 99% (438 of 441) were niacin positive and 97% (426 of 441) were nitrate positive, while among the isolates of the Asian strain tested, 100% (52 of 52) were niacin positive and 88% (46 of 52) were nitrate positive. The oxygen preferences of the two groups also differed slightly, with 100% (38 of 38) of the isolates of the classical strain and 94% (49 of 52) of the isolates of the Asian strain preferring aerophilic conditions. These results demonstrate that the assay is capable of rapidly identifying *M. tuberculosis* isolates, even those with an atypical phenotype.

Of the six isolates identified as *M. africanum* on the basis of the presence of RD 1, the absence of RD 9, and variable results for RD 3, RD 5, RD 10, and RD 11, 100% (six of six isolates) were susceptible to TCH and PZA and preferred microaerophilic conditions. In addition, 83% (five of six) were niacin positive and 33% (two of six) were nitrate positive.

As was the case for the *M. bovis* and *M. bovis* BCG isolates among the original 88 isolates tested (Table 3), *M. bovis* and *M. bovis* BCG were the easiest to differentiate from the other members of the TBC. The 8 isolates of *M. bovis* (which were positive for RD 1 and variable for RD 3, with RD 5, RD 9, RD 10, and RD 11 being absent) and the 13 isolates of *M. bovis* BCG (from which all six regions were absent) were all susceptible to TCH and resistant only to PZA. These variants differed in their oxygen preferences, with *M. bovis* preferring microaerophilic conditions and *M. bovis* BCG preferring aerophilic conditions.

DISCUSSION

The housekeeping genes and genes encoding immunogenic proteins are highly conserved among members of the TBC (24, 31), such that commercially available nucleic acid probes and amplification assays cannot differentiate the different organisms in the complex. Comparative genomics of the members of the TBC by use of subtractive hybridization (22), bacterial artificial chromosome arrays (3, 11), or DNA microarrays (2) identified 16 regions ranging in size from 2 to 12.7 kb that were present in *M. tuberculosis* H37Rv and absent in most BCG derivatives and also in other members of the TBC. These results suggested that deletion of genomic regions has been important in generating genetic diversity within the complex (4, 18, 26). Deletions often arise from recombination between insertion sequence (IS) elements (5, 9), and the *M. tuberculosis* genome contains greater than 40 ISs and mobile genetic elements that could mediate deletions (6). Furthermore, the presence of variable regions in members of the TBC suggested that this approach could be used as a tool for differentiation of members of the TBC. On the basis of the results of these studies, we sought to validate the use of deletion analysis as a solution to the problem faced by clinical mycobacteriology laboratories for differentiation of the members of the TBC.

The junction sequences flanking the variable regions used in this study suggest that at least two different mechanisms are responsible for the deletions. First, RD 3, RD 5, and RD 11 all contain mobile genetic elements (prophage ϕRv1, insertion element IS*6110*, and prophage ϕRv2, respectively). The distribution of these regions in the members of the TBC can be variable. In contrast, the junction regions bordering RD 1, RD 9, and RD 10 do not contain repetitive sequences; these deletions occur in coding regions and result in the truncation of genes (11). The exact mechanism for this type of deletion remains obscure, but DNA polymerase slippage errors may be responsible.

Because of the conservation of junction sequences flanking RD 1, RD 9, and RD 10, multiprimer PCR assays were used to detect these regions. Three primers were included in each assay; two primers were specific for the sequences that flanked the region, and the third was specific for an internal sequence close to one of the flanking primers. Thus, the size of the PCR

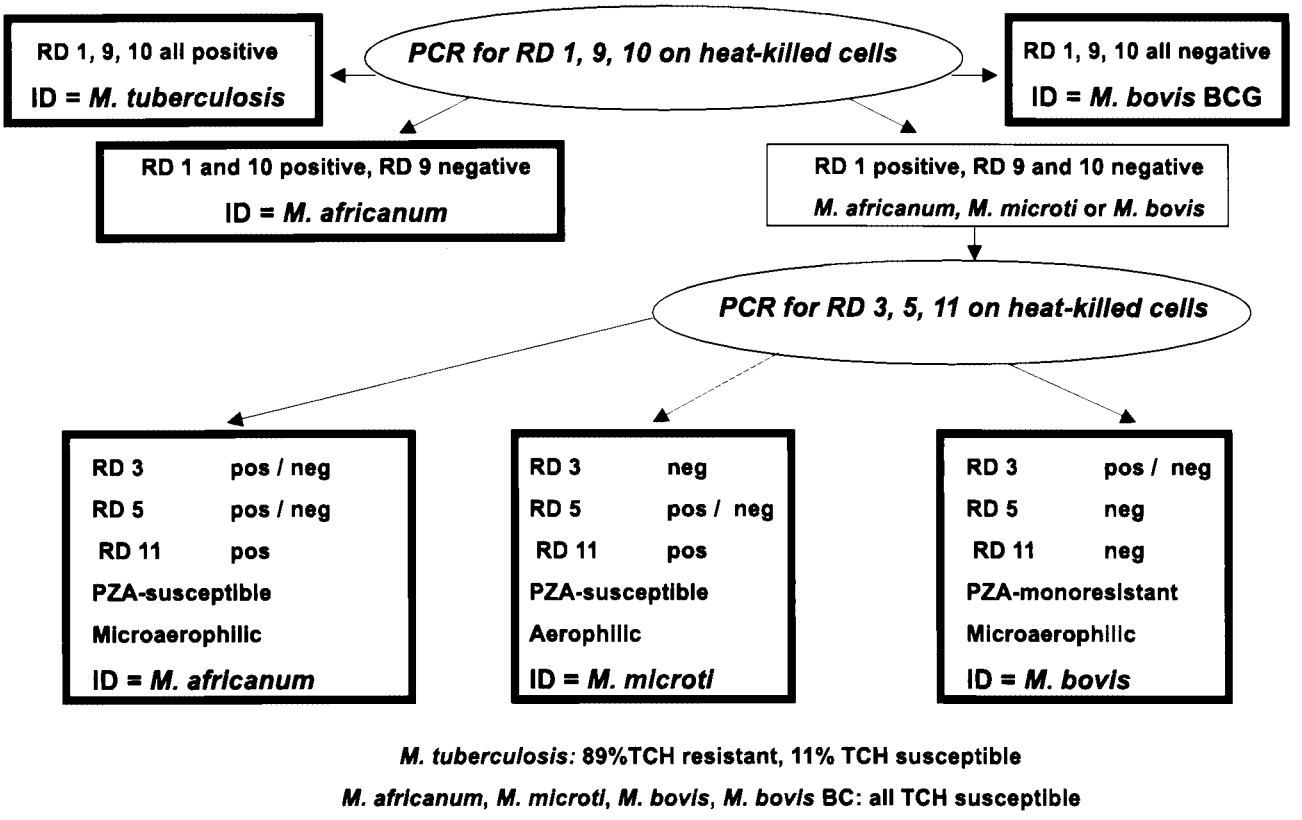

FIG. 3. Flow chart for identification of TBC isolates by deletion analysis.

product was used to determine the presence or the absence of these regions. In contrast, the junction sequences flanking variable regions RD 3, RD 5, and RD 11 are not highly conserved because these regions contain mobile genetic elements whose distributions can differ. Thus, only two primers complementary to internal regions were used for these assays.

First, 88 well-characterized members of the TBC were tested for the presence or the absence of six regions located in various parts of the chromosome. Specific deletion profiles were found for each of the members of the TBC (Table 3), with only *M. tuberculosis* strains containing regions RD 1, RD 9, and RD 10. Significantly, a recent study on the evolution of the members of the TBC also found that the sequences of *M. tuberculosis* strains are highly conserved with respect to RD 1, RD 9, and RD 10 and that these three regions can be used to differentiate *M. tuberculosis* strains from the other members of the TBC (4).

On the basis of the results presented here, a new approach (Fig. 3) was used to rapidly differentiate 605 members of the TBC isolated from patient specimens (Table 4). This approach used prescreening of the isolates by three PCR assays (for RD 1, RD 9, and RD 10). When all three regions were present, the isolate was identified as *M. tuberculosis*. When all three regions were absent, the isolate was identified as *M. bovis* BCG. Isolates that lacked only RD 9 were identified as *M. africanum*, a result also reported in a recent study (4). Those lacking both RD 9 and RD 10 were tested for the presence of RD 3, RD 5, and RD 11; and the results were used to identify *M. bovis* and *M. africanum* (and potentially *M. microti*). This approach provided rapid identifications of members of the TBC that were later confirmed by slower conventional assays, such as TCH and PZA susceptibility tests and assays for oxygen preference, niacin accumulation, and nitrate reductase activity.

This study has opened new perspectives for the rapid identification of individual strains of the TBC in the clinical laboratory. Although more than 90% of the isolates encountered in this study were identified as *M. tuberculosis*, the results suggest that it would be advantageous to use this approach in areas of the world where unusual members of the TBC are more common. The application of the pathway presented here might become even more powerful by including an assay for the *M. tuberculosis*-specific deletion (TbD1) that was identified only recently (4). Finally, we propose that this PCR-based approach, which is simple to perform, can be incorporated into the laboratory routine by many clinical mycobacteriology laboratories. While assays requiring a multistep hybridization technique such as spoligotyping or DNA sequencing may not be within the scope of a clinical diagnostic laboratory, many laboratories use amplification procedures. In our experience, this approach has provided a means for the rapid and clear differentiation of members of TBC and determination of the prevalence of each of the members within the population in New York State. Moreover, most of these isolates are clinically significant, and patient treatment is dependent on their correct and timely identification, suggesting that use of this method is likely to enhance primary care and public health services.

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